Vol.49, Special : pp. 41-46, January 2006
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Determination of Beta-Lactam Residues in Milk by High Performance Liquid Chromatography

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ABSTRACT

A high performance liquid chromatographic method to assay beta-lactam residues in milk was developed and validated. Milk samples were spiked with standard solutions and deproteinated. The extract was cleaned-up on C18 SPE cartridge, the antibiotics eluted with acetonitrile:water (50:50 v/v) and derivatized with acetic anhydride and 1-methyl-imidazole solution containing $HgCl_2$. The chromatographic analysis was performed on C18 column using mobile phase consisting of acetonitrile and phosphate buffer (pH 6.5) in the presence of $Na_2S_2O_3$ gradient and detection at 325 nm. The method was selective for ampicillin, penicillin G and penicillin V, the latter used as internal standard. Average recoveries for ampicillin and penicillin G ranged, respectively, from 60.0% to 104.9% and from 82.7% to 109.2%, with coefficients of variation from 11.1% to 24.6%, and from 2.1% to 25.2%, indicating accuracy and precision. Detection limit of 4.0 μg/L for ampicillin and 3.0 μg/L for penicillin G, and quantification limits of 4.0 μg/L for both were estimated.

Key words: Antibiotics, milk, chromatography

INTRODUCTION

The use of antibiotics is a common practice in clinics and in animal production. Residues from those drugs can be present in animal tissues or secretions in a low concentration, and they are strictly connected with food safety and international trade. Many countries regulate the use of antibiotics in food production animals, set up the maximum residue limits (MRL) and monitor their occurrence. The National Residue Control Plan of Animal Origin Products (PNCR) was established by the Ministry of Agriculture in Brazil presuming the adoption of Residues Control Programs of meat, honey, milk and fish. To execute this Plan, the laboratories from private and government sectors must have validated analytical methods to confirm the identity and quantity of the residues (Brasil, 1999).

The chemical analysis of beta-lactam in milk is not an ordinary process. The lactam ring instability toward acids and alkalis shows a problem in the extraction and purification. At the same time, purification phases are necessary to remove the large number of matrix interference before the chromatographic determination (Schenck and Callery, 1998). Moreover, the detection could be a difficult process, as penicillin is not fluorescent

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and does not have any strong UV chromophores, absorbing at 210 nm (Tarbin et al., 1995). Additionally, a large number of endogenous substances in biological fluids, especially in milk, have a good molar absorption around 200 nm (Wiese and Martin, 1989). Moreover, the low level established for residues in milk is a complicated factor.

In the PNCR, the MRL of 4 μg/L has been adopted for the regulation of penicillin G and ampicillin residues in milk. Boison et al. (1994) presented a method by HPLC with UV detection and obtained a detection limit of 3 μg/L. Sorensen et al. (1997) optimized this method and made it possible to simultaneously determine six different kinds of penicillin in milk, among which were ampicillin and penicillin G, with detection limits of 1.3 μg/L and 1.5 μg/L, respectively.

From what was exposed, and based on the immediate necessity to implement control programs to monitor the presence of residues in milk, a method to assay beta-lactam residues in milk was developed and validated.

MATERIALS AND METHODS

Reagents

Derivatization reagent I was prepared by dissolving 95 μL of acetic anhydride in acetonitrile, followed by dilution to 5 mL.

Derivatization reagent II (2 mol/L 1-methyl-imidazole containing 10^{-3} mol/L $HgCl_2$) was prepared by dissolving 4.0 mL of 1-methyl-imidazole (Sigma) in 10 mL of water and adding 2.5 mL of 0.01 mol/L $HgCl_2$ (Mallinckrodt). The pH was adjusted to 6.8 ± 0.2 with H_3PO_4 85% (Grupo Química) and the solution was diluted to 25 mL. Derivatization reagents I and II were prepared 1-4h before use.

Buffer H_3BO_3.KCl - NaOH was prepared by dissolution of 6.18 g of H_3BO_3 and 7.45 g of KCl in water, and dilution to 250 mL. A volume of 185 mL of 2 mol/L NaOH was added to that solution and the mixture was diluted to 500 mL with water.

0.1 mol/L NaH_2PO_4 pH 8.0 was prepared by dissolution of 6.90 g of $NaH_2PO_4.H_2O$ in approximately 400 mL of water, followed by adjustment of pH to 8.0 with 10 mol/L NaOH and 1 mol/L NaOH and dilution to 500 mL.

0.1 mol/L phosphate buffer pH 9 was prepared by dissolution of 0.68 g of KH_2PO_4 in approximately 150 mL of water, followed by adjustment of pH to 9.0 with 5 mol/L NaOH and 1 mol/L NaOH and dilution to 200 mL.

0.1 mol/L phosphate buffer with 0.0157 mol/L $Na_2S_2O_3$, pH 6.5 was prepared by dissolution of 4.97 g of Na_2HPO_4, 9.0 g of $NaH_2PO_4.H_2O$ and 2.48 g of $Na_2S_2O_3$ anhydrous in approximately 800 mL of water, followed by dilution to 1000 mL and filtration using 0.45 μm filter.

McIlvaine buffer pH 4.5 was prepared mixing 500 mL of 0.1 mol/L citric acid with 440 mL of 0.2 mol/L NaH_2PO_4, followed by adjustment of pH to 4.5 with the first solution.

Standard solutions

Stock standard solutions at a concentration of 100 μg/mL of ampicillin (Sigma), penicillin G (Sigma) and penicillin V (Sigma) were prepared individually in distilled and deionized water, previously filtered in 0.45 μm membrane. Mixed standard working solutions containing ampicillin and penicillin G were prepared from the stock solutions at the following concentrations: 0.2 μg/mL; 0.4 μg/mL; 0.6 μg/mL; 0.8 μg/mL; 1.6 μg/mL; 2.0 μg/mL; 2.4 μg/mL; 3.2 μg/mL and 4.0 μg/mL. Penicillin V working standard solution was prepared from the stock solution at a concentration of 2.0 μg/mL. The solutions were stored between -14°C and -20°C for at least one month.

For calibration curve, 100 μL of the mixed standard working solutions were pipetted into glass tubes, to obtain concentrations equivalent to 4; 8; 16; 20, 24, 32 and 40 μg of the antibiotic per liter of milk. Afterwards, 100 μL of 2.0 μg/mL penicillin V solution, 225 μL of phosphate buffer pH 9 and 75 μL of derivatization reagent I were added. The mixture was vortex-mixed and allowed to react for 5 minutes. A 500 μL volume of derivatization reagent II was added to each solution, followed by vortex-mixing and reaction at 70°C for 60 minutes. The solutions were cooled down in cold water and injected in the chromatography system.

Samples

Raw milk samples provided by cows not treated with antibiotics have been obtained from the Ministry of Agriculture Farm in Brazil. Those

samples were used as blank samples in the development and validation of the method.
The gathered samples were stored between -14°C and -20°C and defrosted at room temperature before the analysis. Afterwards, the samples were centrifuged at 1800 x g for 20 minutes at 10°C to separate the fat.
Quotas of 10 mL of defatted milk were pipetted into 50 mL polypropylene centrifuge tubes. For the spiked samples, 100 µL of the mixed standard working solutions was added, to obtain concentration levels identical to the standards. Afterwards, 100 µL of 2.0 µg/mL penicillin V solution (internal standard) was added. The samples were vortex-mixed. A volume of 20 mL of McIlvaine buffer, pH 4.5 was added followed by 5.8 mL of 0.17 mol/L H_2SO_4 and 6.0 mL of 5% sodium tungstate. The mixture was then immediately shaken and the pH was measured. The pH should be between 4.4 and 4.6. If different from that, the process should be restarted with a bigger or smaller volume of 0.17 mol/L H_2SO_4. The mixture was centrifuged at 1800 x g for 20 minutes at 10°C for protein precipitation. The clear supernatant was transferred to a becker and the pH was adjusted between 8.1 to 8.3 with H_3BO_3.KCl - NaOH buffer. The liquid was centrifuged at 1800 x g for 10 minutes at 10°C. After centrifugation, the extract was eluted on a C18 SPE cartridge at a flow-rate between 2 mL and 3 mL per minute. The cartridge was pre-conditioned with 10 mL of methanol followed by 10 mL of water, 5 mL of 2% NaCl and 5 mL of 0.1 mol/L sodium phosphate pH 8.0. After elution, the cartridge was washed with 3 mL of 2% NaCl followed by 3 mL of water, and dried by drawing air through the cartridge for 5 minutes. The antibiotics were eluted with 1 mL of acetonitrile:water (50:50 v/v). The eluate was collected in a 10 mL vial and evaporated to 100 µL under nitrogen-flow at 45°C to 50°C. After evaporation, 325 µL of phosphate buffer pH 9 were added and from then on, the procedure was the same adopted for the standard solutions.

Material and instrumentation

Balance (Mettler AE 160); automated pipettes (Eppendorf); centrifuge (Sorvall Instruments RC-3B); pHmeter (Metrohm 632); water-bath with shaker (Yamato BT 25); vortex mixer (Phoenix Mod. AT56A); tube heater (Kontes); solid phase extraction cartridges C18/14%, 500 mg, 6 mL (Applied Separations); SPE vacuum manifold (Vac-elut Varian SPS 24); high performance liquid chromatography system (Thermo Separation Products - TSP).

LC analysis

Mobile phase consisting of 0.1 mol/L phosphate buffer (pH 6.5) containing 0.0157 mol/L $Na_2S_2O_3$ and acetonitrile (Vetec) was used according to the following gradient flow: 0 minute (90:10), 5 minutes (90:10), 15 minutes (75:25), 25 minutes (65:35), 27 minutes (90:10) and 30 minutes (90:10), at a flow-rate of 1.0 mL/minute. A Lichrospher - 100 RP 18, 25 cm x 4 mm, 5 µm, (Merck) column was used. The injection volume was 200 µL and the detection performed at 325 nm.

Calculation

The quantification of analytes was carried out injecting standard solutions, blank sample and spiked samples. For each beta-lactam analyzed, the factor of response consisting of the ratio between the height of the analyte peak and the height of the internal standard (penicillin V) was verified.

Validation parameters

For linearity studies, curves with seven levels were built, ranging from 4 µg/L to 40 µg/L. The samples were analyzed in duplicate, in two different days. To verify the selectivity, the resolution of the peaks, the presence of interference and the concordance in retention times of blank samples and spiked samples were observed. Accuracy and precision were studied by analyzing samples spiked to concentrations levels of 4 µg/L, 6 µg/L and 8 µg/L, with a minimum of six repetitions per level, in three different days. The average recovery and the coefficient variation were calculated for each concentration and compared with the acceptable. Codex (1993) and Brasil (2003) have determined for the concentration between 1 µg/L and 10 µg/L, the acceptable range of recovery between 60% and 120% with maximum variation coefficient of 30%. The quantification limit was determined by the minor amount of analyte detected in 10 studied repetitions which contemplated the values of recovery percentage and coefficient of variation acceptable (Eurachem, 1998). The detection limit has been determined by the minor amount of

analyte detected in the 10 repetitions even though they have not necessarily granted the acceptable criteria.

RESULTS AND DISCUSSION

Development of the method

The technique for protein precipitation using 0.17 mol/L H_2SO_4 and 5% sodium tungstate was fast, easy and efficient, with the best results between pH 4.4 and 4.6. The use of McIlvaine buffer pH 4.5 made the pH stabilization easier on the recommended range. The adjustment of the extract pH between 8.1 and 8.3, before the elution, was important to avoid clogging of C18 cartridge (Boison and Keng, 1998). The use of C18 SPE cartridge for purification of extracts containing beta-lactams has been described by many authors (Terada and Sakabe, 1985; Munns et al., 1985; Boison et al, 1994; Tarbin et al., 1995; Sorensen et al., 1997; Luo et al., 1997; Boison and Keng, 1998; Takeba et al., 1998) and was also appropriate in this method. The 1 mL volume of acetonitrile:water 50:50 v/v was sufficient to extract the beta-lactams completely. The eluate collected after a second addition of that mixture did not show beta-lactams peaks in its respective chromatogram.

The strategy for the simultaneous analysis of monobasic and dibasic penicillin has relied on the choice of reaction conditions that favor formation of stable products by both dibasic and monobasic penicillin, with the same degree of specificity, in accordance to the mechanism proposed by Bundgaard (1974). In the developed method, the amphoteric properties of ampicillin were removed by derivatization of the -NH2 with acetic anhydride. The reaction with 1-methyl-imidazole allowed the penicillin to form stable products with absorption maxima at 325 nm (Bundgaard and Ilver, 1972; Bundgaard, 1974). The use of a higher UV-region reduced the impact of other substances. The reaction optimized conditions were pH 6.8 ± 0.2, 70°C for 60 minutes.

A mobile phase consisting of acetonitrile and 0.1 mol/L phosphate buffer, pH 6.5 in the presence of 0.0157 mol/L $Na_2S_2O_3$ gradient, at a flow-rate of 1.0 mL/minute was appropriate for the chromatography analysis.

Validation of the method

The method has presented linearity on the concentration range from 4 μg/L to 40 μg/L for the antibiotics in milk, which was demonstrated by linear calibration curves and coefficient of determination over 0.99. The response factors of the calibration curves for ampicillin and penicillin G were compared in two different days by the test, presuming equivalent variances, and significant difference ($p < 0.05$) between both days was not observed. In the method developed by Boison et al. (1994) calibration curves plotted from the response ratios of Penicillin G/Penicillin V were linear between concentrations of 3 μg/L and 60 μg/L, with a correlation coefficient higher then 0.999. In the method developed by Sorensen et al. (1997), calibration curves were linear for the antibiotics tested with correlation coefficients between 0.995 and 0.999, in the range from 4 μg/L to 45 μg/L.

In the validated method, selectivity for ampicillin, penicillin G and penicillin V was obtained, evident by the following: complete separation of these analytes from the matrix interferences, peak resolution and satisfactory recoveries.

The average recoveries for each studied concentration ranged from 60.0% to 104.9% for ampicillin, and from 82.7% to 109.2% for penicillin G, granting the recommended recovery range (Codex, 1993; Brasil, 2003) and demonstrating the accuracy of the method. For the evaluation of precision, the coefficients of variation have been calculated for each concentration level, on the same day and in three different days. On the same day, the coefficients of variation ranged from 13.8% to 24.6%, from 11.6% to 16.8% and from 11.1% to 20.5% for ampicillin, and from 5.9% to 17.8%, from 5.3% to 25.2% and from 2.1% to 13.3% for penicillin G, respectively in the concentrations of 4 μg/L, 6 μg/L and 8 μg/L. In different days, the coefficients of variation were of 22.3%, 28.9% and 17.3% for ampicillin and from 18.0%, 12.0% and 9.6% for penicillin G, respectively in the concentrations of 4 μg/L, 6 μg/L and 8 μg/L. These results confirm the precision of the method in accordance with the recommended criteria (Codex, 1993; Brasil, 2003).

The detection limits determined were of 4 μg/L and 3 μg/L, for ampicillin and penicillin G respectively. The detection limit for penicillin G in the method developed by Boison et. al. (1994) was

of 3 μg/L for penicillin G. The detection limits developed by Sorensen et al. (1997) were of 1.4 μg/L for ampicillin and of 1.3 μg/L for penicillin G. The quantification limits of the method coincided with those from detection, granting the MRL allowed by the Brazilian and international legislation.

Conclusion

The method by HPLC presented in this work is adequate to determine residues of ampicillin and penicillin G in milk, and the quantification limits obtained grant the Brazilian and international legislation.

ACKNOWLEDGMENT

We thank LANAGRO/MG and Post Graduation in Pharmaceutical Sciences Program from Faculdade de Farmácia UFMG for the support.

RESUMO

Um método para determinar resíduos de antibióticos beta-lactâmicos em leite por cromatografia líquida de alta eficiência (CLAE) foi desenvolvido e validado. Amostras brancas foram adicionadas de padrão e desproteinizadas. O extrato foi purificado por extração em fase sólida C18, os antibióticos eluídos com acetonitrila:água (50:50 v/v) e posteriormente derivatizados com anidrido acético e solução de 1-metil-imidazol contendo $HgCl_2$. A análise cromatográfica foi realizada utilizando coluna C18, fase móvel composta por acetonitrila e tampão fosfato pH 6,5, na presença de $Na_2S_2O_3$ em gradiente e detecção a 325 nm. O método foi seletivo para ampicilina, penicilina G e penicilina V, sendo este último utilizado como padrão interno. As médias de recuperação para ampicilina e penicilina G situaram-se, respectivamente, na faixa de 60,0% a 104,9% e de 82,7% a 109,2%, com coeficientes de variação na faixa de 11,1% a 24,6%, e de 2,1% a 25,2%, indicando exatidão e precisão. Limites de detecção e quantificação de 4,0 μg/L para ampicilina e de 3,0 μg/L para penicilina G foram determinados.

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Received: July 29, 2005;
Revised: September 05, 2005;
Accepted: November 22, 2005.